First report of potato spindle tuber viroid isolated from pepper seeds produced in Vietnam

Saori Tanaka
Yokohama Plant Protection Station
Ryota Murase
Yokohama Plant Protection Station
Yoshimi Inoue
Yokohama Plant Protection Station
Masumi Masumoto
Yokohama Plant Protection Station
Takayuki Matsuura
Yokohama Plant Protection Station
Hironobu Yanagisawa (hironobu_yanagisa090@maff.go.jp)
Yokohama Plant Protection Station https://orcid.org/0000-0002-6729-466X

Research Article

Keywords: Pepper, seeds, Potato spindle tuber viroid, viability

Posted Date: November 17th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3605685/v1

Version of Record: A version of this preprint was published at Journal of General Plant Pathology on February 15th, 2024. See the published version at https://doi.org/10.1007/s10327-024-01170-8.

Abstract

Potato spindle tuber viroid (PSTVd; genus *Pospiviroid*) was detected from pepper (*Capsicum annuum*) seeds produced in Vietnam, during an export inspection. The PSTVd isolate maintained its viability. To investigate the phylogenetic relationship between PSTVd-VN and other PSTVd variants isolated from other plants, the PSTVd isolate was classified into lethal and severe strains. Inoculated tomatoes (cv. Rutgers) caused severe stunting, with necrosis of the stems and leaf veins. Although the inoculated pepper plants were asymptomatic, the fruits were smaller than the healthy ones. To our knowledge, this is the first report of PSTVd from pepper seeds produced in Vietnam.

Full Text

Viroids are unencapsidated, single-stranded, circular RNA consisting of 246–401 nucleotides. Among plant pathogens, they are the smallest pathogens and belong to two families (Pospiviroidae and Avsunviroidae) (Flores et al. 2005). The genus *Pospiviroid* in the family Pospiviroidae consists of nine species, of which one is the potato spindle tuber viroid (PSTVd) (Full ICTV Report on the Genus *Pospiviroid*; https://ictv.global/report/chapter/pospiviroidae/pospiviroidae/pospiviroid).

Although PSTVd was originally reported in North America, it has spread to Africa, Asia, Europe, and South America (Smith et al. 1997). In recent years, PSTVd has been identified in several countries, and new viroids are being continuously discovered (Kinoga et al. 2021; Matsushita et al. 2021; Sial and Khan 2018).

PSTVd infects various host plants and causes stunting, leaf chlorosis, leaf epinasty, fruit distortion, and stem and leaf necrosis in tomatoes (*Solanum lycopersicum*) and potatoes (*S. tuberosum*) (Hadidi et al. 2003; Singh et al. 2003). Viroids spread mechanically through plant sap, grafting, and transmission through seeds and pollen (Matsushita et al. 2018). Among these, seed transmission is an important factor responsible for the expansion of viroids as reported for PSTVd, which is transmitted through the seeds of *Solanum* spp., such as tomato, pepper (*Capsicum annuum*), and petunia (*Petunia* × *hybrida*) (Matsushita and Tsuda 2016). Moreover, PSTVd has been detected in traded tomato and pepper seeds (Constable et al. 2019), and thus, such viroids can spread to new areas through these contaminated seeds. Therefore, several countries, including Japan, are focusing on the transmission of PSTVd through contaminated seeds. To prevent the occurrence of PSTVd, plant quarantines established in each country require seed testing for viroids. Additionally, viroids have been detected in new plants and countries using these tests. In this study, we present the first report of PSTVd detected in pepper seeds produced in Vietnam, where viroids have not yet been reported. Additionally, we assessed the viability of the viroid in the seeds and its pathogenicity, and conducted phylogenetic analysis.

Pepper seeds from Vietnam were tested to confirm the absence of pospiviroid contamination during export inspections in Japan in 2022. RNA was extracted from seed samples according to the method described by Yanagisawa et al. (2012). To detect pospiviroids in each sample, we used reverse

transcription quantitative real-time polymerase chain reaction (RT-qPCR), as reported by Yanagisawa et al. (2017). Detection tests were conducted in duplicates for each sample. First, primary screening was confirmed to be positive in the assay using SYBR Green with a universal primer set (6Pospi-F/R), which enabled the detection of six pospiviroids, excluding the columnea lant viroid and pepper chat fruit viroid. Ct values were 31.0 and 30.8, and both melting temperature (Tm) values were 86.4 °C. Both Tm values of the positive controls synthesized artificially based on the PSTVd sequence (GenBank/EMBL/DDBJ accession no. EU862231, Matsushita et al. 2010) were 86.0. These results estimated that the viroid in the sample was PSTVd because the Tm values of the samples and the positive control were similar. Moreover, RT-qPCR was performed using TaqMan with specific primer/probe sets for each viroid to identify the pospiviroid species. As the result, Ct values (23.4 and 22.9) were obtained in the PSTVd-specific primer/probe set (PS-F1/SM-R1/P3R), and the primer/probe sets of other viroids did not react. These results confirmed that the viroid species was PSTVd.

To determine the complete sequence of the viroid, direct sequencing analysis of PCR products obtained from the primer sets (6Pospi-F/R and PV68/87), (Yanagisawa et al. 2019) which can amplify the almost complete genome of PSTVd, was conducted in triplicate. The results showed that the complete 359 nt sequence of PSTVd (PSTVd-VN, Acc. No. LC784333) showed the highest identity (98.6%) to a PSTVd variant (No. MT663308) from tomato.

Subsequently, to investigate the phylogenetic relationship between PSTVd-VN and other known PSTVd variants isolated from pepper and other plants, and some PSTVd variants that confirmed pathogenicity, a phylogenetic tree was constructed based on full genome sequences using the Maximum Likelihood method with 1,000 replicates of bootstrapping performed in the MEGA version VII program (Fig. 1). The results showed that PSTVd-VN was grouped into a cluster that included the PSTVd variant (No. MT663308) but not the PSTVd variants previously isolated from pepper. In addition, PSTVd variants (Nos. AY518939; severe, HE575349; severe, and U23058; lethal) with strong pathogenicities existed in the cluster that contained PSTVd-VN.

Next, to confirm whether the detected PSTVd maintained the viability of viroids in seeds, the nucleic acid extracted from seeds was used as the inocula to inoculate in tomatoes (cv. Rutgers) and two varieties of pepper (bell pepper and chili pepper). PSTVd was mechanically inoculated into the young leaves of the seedlings according to the method described by Yanagisawa and Matsushita (2017). At least six seedlings of each plant were inoculated, and the plants were then grown in a greenhouse at 23–25 °C with natural light. Simultaneously, tomato plants were inoculated with two different pathogenic PSTVd variants to compare the pathogenicity. A PSTVd variant (No. AB623143, Tsushima et al. 2011) exhibiting mild pathogenicity was used. The other PSTVd variant (No. EU862231) was used for severe pathogenicity because there was only one base difference between the two PSTVd variants (Nos. EU862231 and LC523663, Matsushita et al. 2021) that showed severe pathogenicity. One month after inoculation, viroid infections were assessed in the uppermost leaves of each inoculated plant. RT-PCRs using the primer sets (6Pospi-F/R and PV68/87) were conducted to confirm viroid infection in each inoculated plant. Direct sequencing analysis was conducted using these amplicons to confirm the viroid

sequences after inoculation. The inoculated tomato plants had viroid-like symptoms, such as severe stunting, leaf chlorosis, and leaf deformation, followed by necrosis of the stems and leaf veins (Fig. 2A, E). In addition, PSTVd-VN-inoculated tomato plants showed severe pathogenicity, as the PSTVd variant (No. EU862231), and both PSTVd variants caused necrosis of the stem and leaf veins. Contrastingly, the PSTVd variant (No. AB623143) caused slight stunting. However, neither cultivar of inoculated pepper plants exhibited symptoms (Fig. 3A, B). However, as the inoculated chili pepper plants continued to grow for 3 months after inoculation, the symptoms of the fruits were confirmed. The fruits collected from PSTVd-infected pepper plants were smaller than those from healthy plants, and the yield was eventually reduced (Fig. 3C). The results of direct sequencing analysis showed that the sequence of PSTVd detected in pepper seeds and the sequence of PSTVd after inoculation completely matched.

To the best of our knowledge, this is the first report of PSTVd detection in Vietnamese plants. PSTVd-VN belonged to a cluster (Fig. 1) that includes the PSTVd variant (No. U23058) exhibiting lethal pathogenicity, and PSTVd variants (Nos. AY518939 and HE575349) showing severe pathogenicity. Tomato plants inoculated with PSTVd-VN showed severe symptoms similar to those of the PSTVd variant (No. EU862231) (Fig. 2B, F). Therefore, PSTVd-VN was confirmed to be strongly pathogenic. Additionally, PSTVd-VN-infected pepper plants did not show any symptoms; however, fruit downsizing was observed (Fig. 3B, C). Verhoeven et al. (2020) reported that PSTVd-infected *C. annuum* grew and obtained yields similar to those of healthy plants. Therefore, fruit symptoms may depend on the pepper cultivar, and the PSTVd strain and variant. However, in the field, identifying viroid-infected plants would be difficult because no symptoms are seen in pepper plants.

We confirmed the viability of PSTVd in the pepper seeds (Fig. 2A, E). Verhoeven et al. (2021) confirmed the viability of the viroids in the pepper seeds but did not confirm the viability of the viroids in the tomato seeds. Hence, the viability of viroids in pepper seeds may be more easily maintained than in tomato seeds. However, Matsushita and Tsuda (2016) reported transmission through pepper seeds, while Verhoeven et al. (2020, 2021) did not. Many factors are responsible for the seed transmission of viroids (host plant species and cultivars, viroid species and strain, infection stage, environmental conditions, and distribution of viroids in seed parts) (Matsushita et al. 2018). Infection of the ovule is the key to establishing viroid seed transmission. Because the Ct values of RT-qPCR were low, many viroids were suggested to exist in the tested seeds. Moreover, we confirmed the viability of viroids in seeds. These results suggested that the seeds have the potential to promote transmission of viroids. Therefore, continuous testing for viroids is necessary to determine the distribution of healthy seeds.

Declarations

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no competing financial interests or personal relationships that may have influenced the work reported in this study.

Acknowledgments

We thank our colleagues Daisuke Niwamoto and Hiromasa Yamaguchi for their active participation in our research and for cooperating with us.

References

1. Constable F, Chambers G, Penrose L, Daly A, Mackie J, Davis K, Rodoni B, Gibbs M (2019) Viroid-infected tomato and capsicum seed shipments to Australia. Viruses 11:98
2. Flores R, Hernández C, Martínez de Alba AE, Daròs JA, Di Serio F (2005) Viroids and viroid–host interactions. Annu Rev Phytopathol 43:117–139
3. Kinoga MN, Kuria PK, Miano DW, Wasilwa LA (2021) First report of *Potato spindle tuber viroid* infecting tree tomato in Kenya in mixed infection with Potato virus Y. New Dis Rep 44:E12029
4. Hadidi A, Flores R, Randles JW, Semancik JS (Eds.) (2003) Viroids. Collingwood: CSIRO
5. Matsushita Y, Usugi T, Tsuda S (2010) Development of a multiplex RT-PCR detection system for *Potato spindle tuber viroid* and *Tomato chlorotic dwarf viroid*. Eur J Plant Pathol 128:165–170
6. Matsushita Y, Tsuda S (2016) Seed transmission of *potato spindle tuber viroid*, *tomato chlorotic dwarf viroid*, *tomato apical stunt viroid*, and *Columnea latent viroid* in horticultural plants. Eur J Plant Pathol 145:1007–1011
7. Matsushita Y, Yanagisawa H, Khiutti A, Mironenko N, Ohto Y, Afanasenko O (2021) Genetic diversity and pathogenicity of potato spindle tuber viroid and chrysanthemum stunt viroid isolates in Russia. Eur J Plant Pathol 161:529–542
8. Matsushita Y, Yanagisawa H, Sano T (2018) Vertical and horizontal transmission of Pospiviroids. Viruses 10:706
9. Sial ZK, Khan F (2018) First report on *Potato Spindle Tuber Viroid* (PSTVd) from field grown infected Potato plants (*Solanum tuberosum*) in Pakistan. Biologia (Pakistan) 64:241–246
10. Smith IM, McNamara DG, Scott PR, Holderness M (1997) EPPO/CABI quarantine pests for Europe
11. Singh RP, Ready KFM, Nie X (2003) Viroids of solanaceous species. *Viroids*:125–133
12. Tsushima T, Murakami S, Ito H, He YH, Raj APC, Sano T (2011) Molecular characterization of *Potato spindle tuber viroid* in dahlia. J G Plant Pathol 77:253–256
13. Verhoeven JTJ, Koenraadt HMS, Jodlowska A, Huner L, Roenhorst JW (2020) Pospiviroids infections in *Capsicum annuum*: Disease symptoms and lack of seed transmission. Eur J Plant Pathol 156:21–29
14. Verhoeven JTJ, Botermans M, Schoen R, Koenraadt H, Roenhort J (2021) Possible overestimation of seed transmission in the spread of pospiviroids in commercial pepper and tomato crops based on large-scale grow-out trials and systematic literature review. Plants 10:1707

15. Yanagisawa H, Tsutsumi N, Ishii K, Shiki Y, Kurihara K (2012) Modification on detection method of *Potato spindle tuber viroid* using RT-LAMP. Res Bul. Pl Prot Japan 48:7–12
16. Yanagisawa H, Sano T, Hase S, Matsushita Y (2019) Influence of the terminal left domain on horizontal and vertical transmissions of tomato planta macho viroid and potato spindle tuber viroid through pollen. Virology 526:22–31
17. Yanagisawa H, Shiki Y, Matsushita Y, Ooishi M, Takaue N, Tsuda S (2017) Development of a comprehensive detection and identification molecular based system for eight pospiviroids. Eur J Plant Pathol 149:11–23
18. Yanagisawa H, Matsushita Y (2017) Host ranges and seed transmission of *Tomato planta macho viroid* and *Pepper chat fruit viroid*. Eur J Plant Pathol 149:211–217

Figures

Figure 1

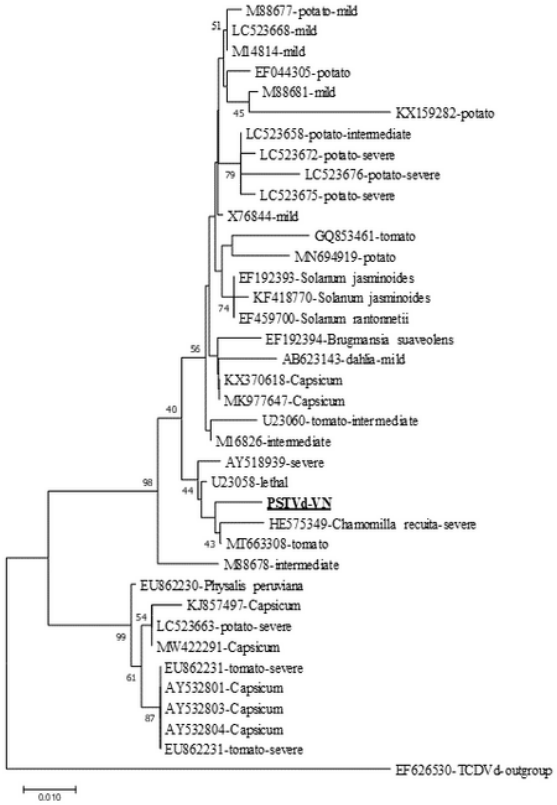

Figure 1

Phylogenetic tree analysis of PSTVd (PSTVd-VN) detected from pepper seeds produced in Vietnam and other PSTVd variants. PSTVd-VN is underlined and bold. Each PSTVd variant described for pathogenicity, such as lethal, severe, intermediate and mild, is a variant that has been investigated for the pathogenicity so far. The tree was constructed using MEGA version 7 based on maximum likelihood (ML) method with 1,000 bootstrap replicates. Numbers at internal nodes indicate bootstrap percentages and the scale bar indicates. Bootstrap values of >40% are indicated at each node. 0.01 substitutions per site.

Figure 2

Figure 2

Symptom of tomato (cv. Rutgers) inoculated with potato spindle tuber viroid (PSTVd) at one month after inoculation. A, E; PSTVd variant (PSTVd-VN; accession No. LC784333) isolated from pepper seeds produced in Vietnam. B, F; PSTVd variant (severe strain; No. EU862231). C, G; PSTVd variant (mild strain; No. AB623143). D, H; healthy plant.

Figure 3

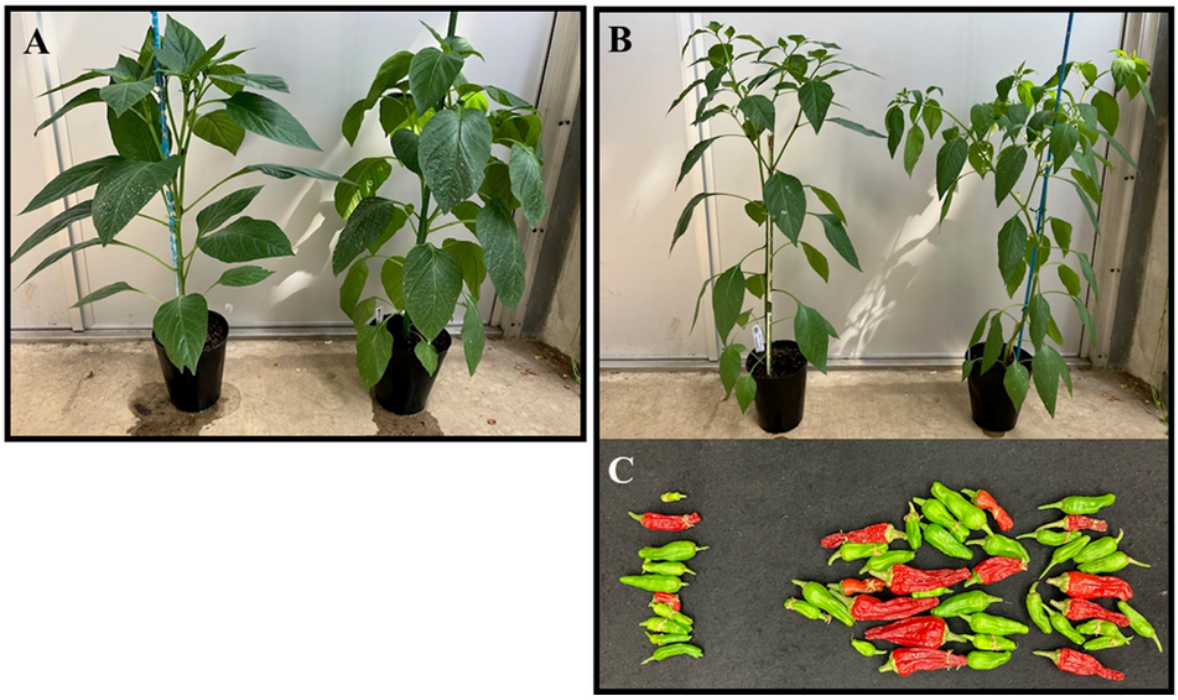

Figure 3

Symptom of pepper plant inoculated with PSTVd at one month after inoculation. A; Bell pepper plants. B; Chili pepper plants. C; Fruits obtained from chili pepper plants at 3 months after inoculation. Left; PSTVd variant (PSTVd-VN, accession No. LC784333) isolated from pepper seeds produced in Vietnam. Right; healthy plant.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- PSTVdVNCompletegenomueAccessionNo.LC784333.docx